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Radioassays of blood group M, N and T (Thomsen–Friedenreich) antigens

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Summary. Radioassays employing the double-antibody or Farr techniques were developed for the M, N and T antigens. Blood group glycoproteins were isolated by butanol extraction of red cell stroma and iodinated by the chloramine-T technique. The final purity of glycoprotein was over 75% as judged by radioimmunoassay (RIA). T activation of glycoprotein was obtained with neuraminidase. A specific RIA was obtained for the M antigen and was sensitive to approximately 10 ng of glycoprotein or glycopeptide. In the RIA system rabbit anti-M displayed a higher affinity for M glycoprotein than for M glycopeptide. A RIA that was entirely specific for the N antigen, could not be obtained. A radioassay, obtained for the T antigen with peanut agglutinin in the Farr technique, was sensitive to approximately 100 ng of T antigen and was readily inhibitable by monosaccharides. A RIA, obtained for the T antigen with rabbit anti-T, was entirely specific and sensitive to approximately 1 ng of T activated glycoprotein or glycopeptide but was not inhibitable by monosaccharides.

INTRODUCTION

The M and N blood group antigens are located on the major sialoglycoprotein (glycophorin A, PAS-1) of the red cell membrane. Immunological activity is dependent upon the integrity of alkali-labile oligosaccharide side-chains. Opinion is, however, divided as to whether the primary structural difference between the M and N antigens is a function of the glycose moiety (evidence reviewed by Springer, Desai, Young and Murphy, 1977) or of the amino acid sequence (Wasniowska, Drzeniek & Lisowska, 1977; Dahr, Uhlenbruck, Janssen & Schmalisch, 1977) The Thomsen–Friedenreich (T) antigen is determined by the structure Gal β (1–3) GalNAc (Uhlenbruck, Pardoe & Bird, 1969) and is the immediate biosynthetic precursor of the M and N antigens (Springer & Desai, 1976).

There is evidence that the MN antigens may also exist on human leucocytes (Gurner & Coombs, 1958; Kosiakov & Urinson, 1959; Stolz, Streiff & Genetet, 1974), spermatozoa (Edwards, Ferguson & Coombs, 1964), tissue cells in culture (Dawson, 1969), in solid organs including muscle and brain (Kosjakov & Tribulev, 1939), liver, spleen and kidney (Boorman & Dodd, 1943) and, more recently, in breast tissue (Springer, Desai & Scanlon, 1976). The T antigen is not normally exposed in healthy tissues (Springer *et al.*, 1976).

Interest in the MN and precursor antigens has recently grown with the report by Springer *et al.*, (1976) that the T antigen is demonstrable in human malignant breast tissue and that anti-T antibody, which is present in all normal human sera, is severely depressed in a significant number of patients with cancer of the breast. Depression of anti-T may be due to binding of antibody to T antigen on the surface of intact tumour cells or to antigen shed from the tumour

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cell membrane (Springer *et al.*, 1977; Anglin, Lerner & Nordquist, 1977). This communication describes the development of sensitive radioassays for the detection of the M, N and T antigens in body fluids.

MATERIALS AND METHODS

Antisera

Rabbit anti-M and anti-N and Vicia graminea extract were generously provided by Dr E. W. Ikin and rabbit anti-T by Professor F. Stratton.

Absorption of antisera

One hundred microlitres of anti-M were diluted with 700 μl of radioimmunoassay (RIA) buffer and absorbed for 2 h at 4° with 100 μl of group M or N red cells. Ten microlitres of rabbit anti-T were diluted with 2·0 ml RIA buffer and absorbed with 100 μl of untreated or neuraminidase treated group M or N cells.

Haemagglutination inhibition

Rabbit anti-M, anti-N and Vicia graminea were diluted 1 in 4 for use. PNA was diluted 1 in 10,000. Serial two-fold dilutions (25 μl) of M, N and T active glycopeptides and glycoproteins (2 mg/ml) were made in microtitre plates. Twenty-five microlitres of diluted antiserum were added to each well and the mixture was incubated at 20° for 60 min. Twenty-five microlitres of a 2% cell suspension were then added, mixed and incubated at 20° for 60 min. The end point was taken as that dilution at which complete inhibition of agglutination was obtained.

Purification of M and N active glycopeptides and glycoproteins

Glycopeptides were prepared by the method of Jackson, Segrest, Kahane & Marchesi (1973) from washed red cells freshly drawn from single group O M, MN and N donors. Glycoproteins were extracted with *n*-butanol (Anstee & Tanner, 1974) from red cell stroma prepared from single donors (Dodge, Mitchell & Hanahan, 1963).

Iodination

Glycopeptides and glycoproteins were iodinated by the chloramine-T technique. Labelled glycoproteins were further chromatographed on Sepharose CL 6B and five radioactive peaks were obtained. The first peak, which eluted shortly after the void volume, contained the most immunologically reactive material.

Desialation of glycopeptides and glycoproteins

Unlabelled or ^{125}I-labelled glycopeptides, glycoproteins and fetuin (Sigma) were desialated with neuraminidase or with acid. Incubation with neuraminidase proceeded for 4 h at 37° and the reaction was terminated by heating in boiling water for 1 min. Hydrolysis in 0·05 N H_2SO_4 proceeded for 60 min at 80° and the hydrolysate was then cooled to 4° and neutralized with 1·0 N NaOH.

T activation of red cells

Five hundred microlitres of washed red cells were incubated with 25 units of neuraminidase in 5 ml of phosphate buffer pH6 containing 0·02 M PO_4, 0·15 M NaCl and 0·01 M $CaCl_2$.

Radioimmunoassay (double-antibody) technique

A suitable dilution of antibody was determined from antibody dilution curves (Fig. 1). Labelled glycopeptide or glycoprotein was diluted in phosphate buffer pH 7·4 containing 0·05 M PO_4, 0·1 M NaCl, 1 g/l Na_4 EDTA and 5 g/l bovine albumin to give approximately 30,000 c.p.m. per 100 μl. Equal volumes (100 μl) of diluted antibody and diluted labelled glycopeptide or glycoprotein were added to 500 μl of phosphate buffer, mixed and incubated overnight at 4°. To this was added 100 μl of a second antibody containing a 1/400 dilution of normal rabbit serum and 1/50 dilution of donkey anti-rabbit immunoglobulin (Burroughs-Wellcome). The mixtures were again incubated overnight at 4°, centrifuged at 1600 ***g*** for 30 min and the precipitates were kept for counting. The non-specific binding of labelled glycopeptide and glycoprotein amounted to 3–6% of the total counts added.

In the assay procedure antibody was used at a dilution at which 30–40% of the radioactivity was bound. One hundred microlitres of diluted antibody and 100 μl of unlabelled glycopeptide or glycoprotein standard or of serum were mixed with 400 μl of phosphate buffer and incubated overnight at 4°. One hundred microlitres of diluted labelled glycopeptide or glycoprotein were then added and the mixture was incubated overnight at 4° before the addition of the second antibody.

Radioassay with peanut agglutinin (PNA)

PNA was purified by the technique of Terao, Irimura & Osawa (1975). A modification of the Farr technique (1958) was used. The assay buffer contained 0·05 M phosphate pH 7, 0·02 M NaCl, 2 g/l bovine albumin and 5 g/l IgG prepared from normal human serum

obtained from group AB donors. In a standard reaction mixture, 200 μl PNA dilution and 200 μl of diluted labelled glycoprotein or glycopeptide were incubated for 2 h at 4°. For precipitating lectin-bound labelled glycoprotein, 400 μl of saturated ammonium sulphate were added to the mixture. For precipitating lectin-bound labelled glycopeptide, 600 μl of saturated ammonium sulphate were added. The non-specific binding of labelled T glycopeptide was only about 3–5%, but non-specific binding of labelled T glycoprotein amounted to 45%. Labelled glycoprotein was brought to 50% saturation with ammonium sulphate and the labelled material which co-precipitated with human IgG was discarded. By this technique the non-specific binding of residual labelled glycoprotein was reduced to 25%.

RESULTS

Haemagglutination inhibition

Table 1 records the inhibition of anti-M, anti-N and Vicia graminea by M and N glycopeptides or glycoproteins. In each case glycoproteins were more inhibitory than glycopeptides on a weight basis. Neuraminidase-treated M and N glycoproteins were more inhibitory of PNA than were the treated glycopeptides.

Radioassay studies

Binding of ^{125}I-labelled M and N glycopeptides and glycoproteins

Antibody dilution curves. About 75% of labelled M glycoprotein was bound by the most potent rabbit anti-M. Antibody excess was not achieved at the highest concentration of antibody that could be used (Fig. 1) and the purity of the glycoprotein is, therefore,

Table 1. Haemagglutination inhibition titres, as reciprocals, obtained by untreated and neuraminidase-treated M and N glycopeptides and glycoproteins

	Anti-M	Anti-N	Vg	PNA
M Glycopeptide	32	0	0	4*
M Glycoprotein	64	2	2	1024*
N Glycopeptide	0	2	9	8*
N Glycoprotein	0	64	128	1024*

* Neuraminidase treated.

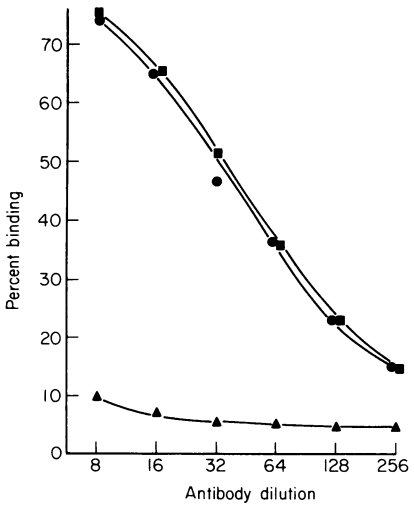

Figure 1. The binding of ^{125}I-labelled M glycoprotein by dilutions of rabbit anti-M unabsorbed (●) and absorbed by group N (■) and group M (▲) red cells.

underestimated. Binding of labelled M glycoprotein by rabbit anti-M could be specifically and completely inhibited with group M red cells. Strong binding of labelled M glycoprotein was obtained by five other rabbit anti-M sera. By contrast only weak binding (25% of radioactivity) of labelled M glycopeptide was detected even at the highest concentrations of rabbit anti-M. Of five rabbit anti-N sera two gave very weak binding of labelled M glycoprotein and no binding was obtained by the other three.

Eight rabbit anti-N sera were tested for binding of labelled N glycoprotein; five anti-N sera gave very weak binding and the best bound only 49% of radioactivity at the highest concentration of antibody that could be used. At the same dilution the best anti-N bound 21% of labelled M glycoprotein and even at the dilution selected for the N assay 15% of labelled M glycoprotein was bound.

Assay of standard MN glycoproteins and glycopeptides. M assay: Unfractionated butanol extracts were used as standard glycoproteins without further purification as chromatography on Phosphocellulose and Sepharose CL 6B failed to improve inhibitory potency. Complete inhibition of rabbit anti-M was obtained by M and MN glycoproteins and the assay was sensitive to approximately 10 ng of M glycoprotein. MN glycoprotein was two- to four-fold less inhibitory than two preparations of M glycoprotein and

the inhibition slopes were parallel (Fig. 2). M glycopeptide and M glycoprotein produced similar magnitudes of inhibition but in two experiments the inhibition slopes obtained with M glycopeptide were significantly shallower than those obtained with M or MN glycoproteins. N glycopeptide was not inhibitory at 1000 ng and N glycoprotein was not inhibitory at 10,000 ng. No inhibition was obtained by neuraminidase-treated M glycoprotein.

N assay: The N assay was sensitive to 2 ng of N glycoprotein. Group M and MN glycoproteins were approximately sixteen- to thirty-two-fold less inhibitory than N glycoprotein (Fig. 3). N glycopeptide was about thirty-two-fold less inhibitory than N glycoprotein. Neuraminidase-treated M and N glycoproteins and N glycopeptides were only weakly inhibitory. The inhibition slopes obtained by N and MN glycoproteins in the N assay were considerably shallower than the slopes obtained by M and MN glycoproteins in the M assay.

Assay of normal human sera. M assay: The effect of 100 μl aliquots of whole normal sera on the uptake of anti-M to labelled M glycoprotein was determined. Marked inhibition of uptake was observed for all sera irrespective of MN blood group. Sera from group M and MN donors gave similar inhibition but both gave more inhibition than sera from group N donors (Fig. 4).

N assay: In the N assay all sera were inhibitory but group MN and N were more inhibitory than M sera (Fig. 4).

In view of the non-specific inhibition of binding results of both assays are expressed as percentage binding. Kosjakov & Tribulev (1939) and Boorman & Dodd (1943) observed non-specific inhibition of anti-M and anti-N by human tissue extracts.

Binding of ^{125}I-labelled T glycopeptide and glycoprotein

Double antibody technique: (i) *Antibody dilution curves.* Immune rabbit anti-T bound 67% of neuraminidase-treated labelled M glycopeptide [T(M) glycopeptide] at the highest concentration used but antibody was not in excess. Absorption of rabbit anti-T by untreated group M or N cells had no effect upon the binding of labelled T(M) glycopeptide but absorption of antibody with neuraminidase-treated M or N cells abolished binding. Untreated labelled M or N glycopeptides were not bound by rabbit anti-T.

(ii) *Assay of standard T glycoprotein and glycopeptides.* An assay was obtained which was sensitive to about 0·5 ng of T(M) glycopeptide and of T(M) glycoprotein (Figs 5 and 6). T(N) glycopeptide was about twice as inhibitory as T(M) glycopeptide irrespective of whether desialation was undertaken with neuraminidase or acid. On a weight basis untreated M and N glycopeptides were 128- to 256-fold less inhibitory

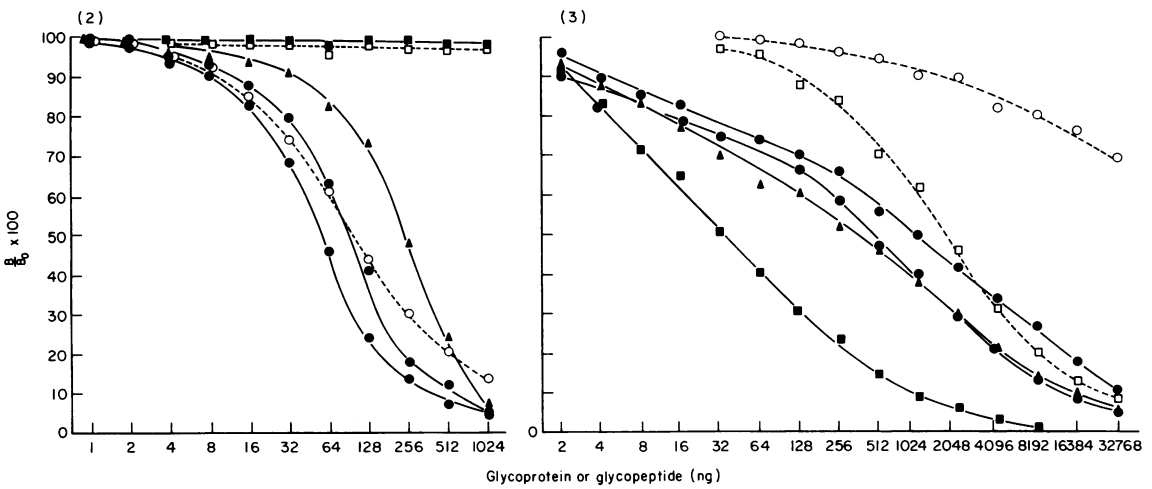

Figure 2. The inhibition of binding of rabbit anti-M to labelled M glycoprotein obtained by M (●), MN (▲) and N (■) glycoproteins and by M (○) and N (□) glycopeptides.

Figure 3. The inhibition of binding of rabbit anti-N to labelled N glycoprotein obtained by M (●), MN (▲) and N (■) glycoproteins and by M (○) and N (□) glycopeptides.

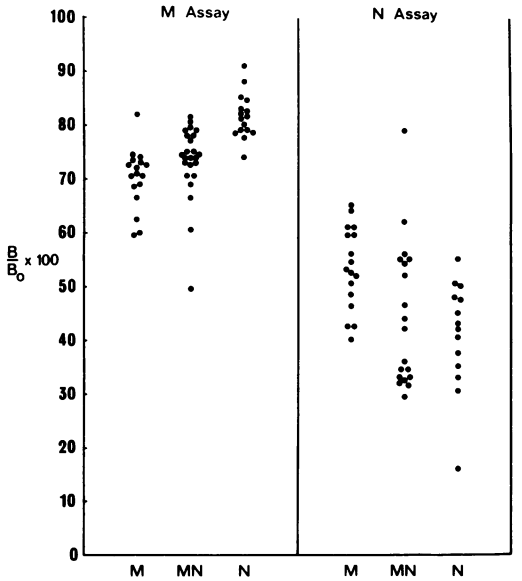

Figure 4. The inhibition of binding obtained in assays for M and N activity by normal sera from group M, MN and N donors.

than the T(M and N) glycopeptides and untreated M and N glycoproteins were about 128-fold less inhibitory than T(M and N) glycoproteins. No inhibition was obtained by untreated or neuraminidase treated fetuin or by D-galactose, N-acetyl-D-galactosamine, D-glucose, N-acetyl-D-glucosamine, lactose, maltose or cellobiose at 80 mM final concentrations.

(iii) *Assay of normal sera.* No significant inhibition was obtained by any of ten normal sera.

Farr technique: (i) *PNA dilution curve.* At an excess of PNA approximately 85% of labelled T(M) glycopeptide or labelled T(M) glycoprotein was bound.

(ii) *Assay of standard glycopeptides and glycoproteins.* Untreated M and N glycopeptides and glycoproteins and fetuin failed to inhibit PNA. T(M) glycoprotein was the most potent inhibitor, 1 μg gave complete inhibition and the assay was sensitive to about 100 ng (Fig. 7). T(N) glycoprotein, T(M) glycopeptide and T(N) glycopeptide were all about two- to four-fold less inhibitory than T(M) glycoprotein. Neuraminidase treated fetuin was eight- to sixteen-fold less inhibitory than T(M) glycoprotein.

(iii) *Inhibition by saccharides.* Figure 8 shows the inhibition of the reaction between PNA and labelled T(M) glycopeptide by monosaccharides. D-Galactose was the most effective inhibitor and D(+)-fucose, which differs only in having a methyl group at the C-6 position, was about two-fold weaker. N-Acetyl-D-galactosamine which differs from D-galactose only at the C-2 position was even more weakly inhibitory

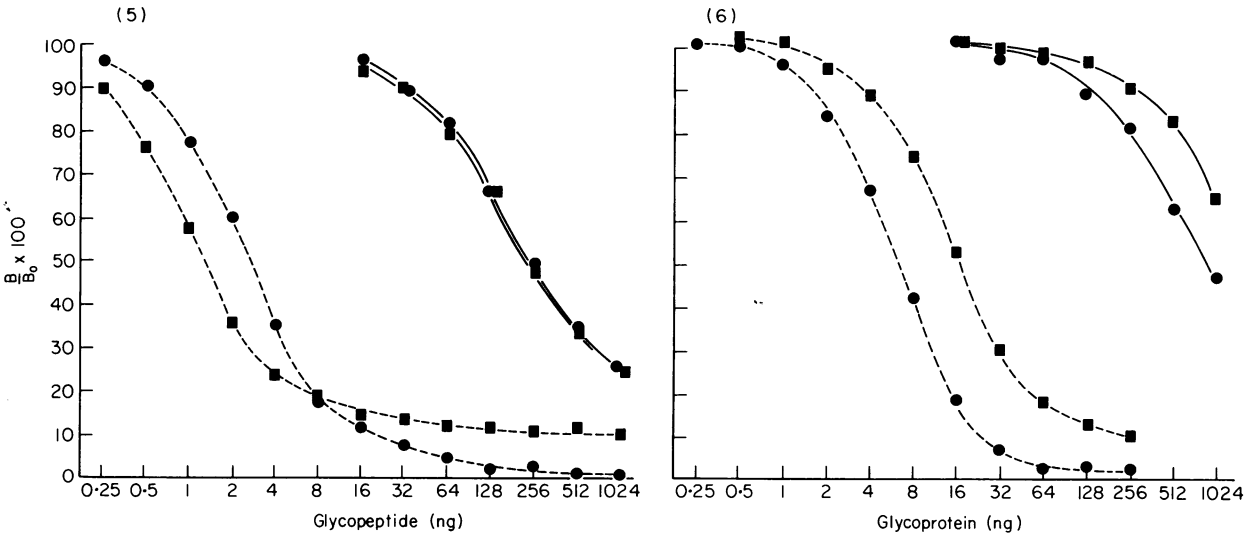

Figure 5. The inhibition of binding of rabbit anti-T to labelled T(M) glycopeptide obtained by untreated (●——●) and neuraminidase-treated (●- - -●) M glycopeptide and by untreated (■——■) and treated (■— — —■) N glycopeptide.

Figure 6. The inhibition of binding of rabbit anti-T to labelled T(M) glycopeptide by untreated (●——●) and neuraminidase-treated (●- - -●) M glycoprotein and by untreated (■——■) and treated (■- - -■) N glycoprotein.

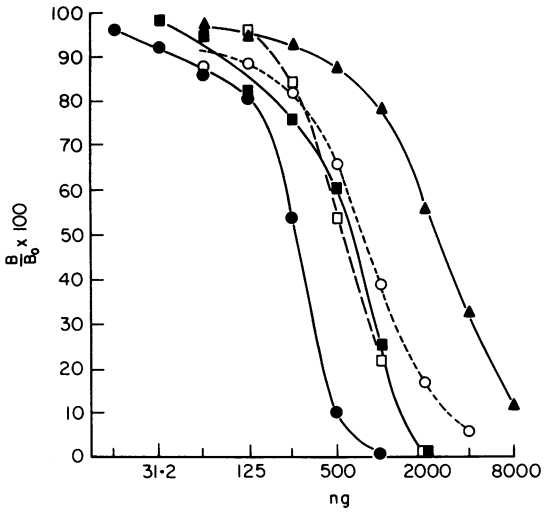

Figure 7. The inhibition of binding of PNA to labelled T(M) glycopeptide by neuraminidase-treated M (●) and N (■) glycoprotein, M (○) and N (□) glycopeptide and by treated fetuin (▲).

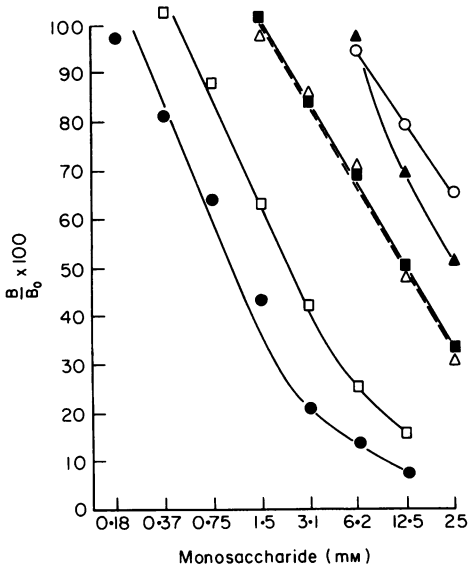

Figure 8. The inhibition of binding of PNA to ^{125}I-labelled T(M) glycopeptide by monosaccharides D-galactose (●), D-fucose (□), N-acetyl-D-glucosamine (△), L-arabinose (■), N-acetyl-D-galactosamine (▲) and D-glucose (○).

confirming the importance of the substitution at the C-2 position (Lotan, Skutelsky, Danan & Sharon, 1975). The weak inhibition obtained by glucose confirmed that the orientation of the hydroxyl group at

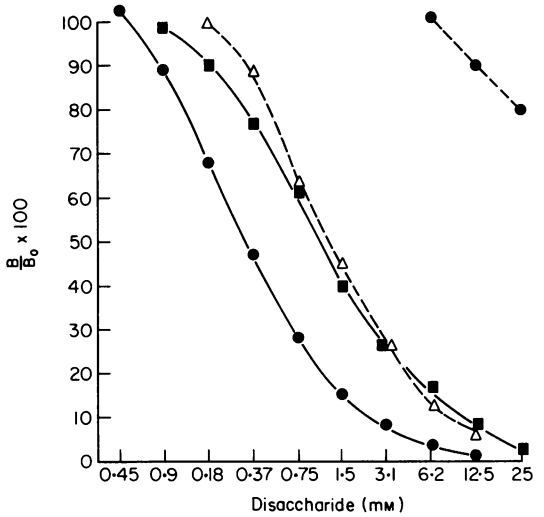

Figure 9. The inhibition of binding of PNA to ^{125}I-labelled T(M) glycopeptide by disaccharides lactose (●——●), melibiose (■), cellobiose (△) and maltose (●– – –●).

C-4 is also very important. Of the disaccharides tested lactose (Galβ(1–4)Glc) was the most inhibitory followed by melibiose (Galα(1–6)Glc) and cellobiose (Glcβ(1–4)Glc). Maltose (Glcα(1–4)Glc) was very weakly inhibitory (Fig. 9). Of disaccharides containing a terminal non-reducing galactose residue, lactose, having a β-linkage, was more inhibitory than melibiose, having an α-linkage. Glucose β-glycosidically linked to a second glucosyl residue was more inhibitory of PNA than the monosaccharide or than the α-glycosidically linked disaccharide. The disaccharide Galβ(1–3)GalNAc was not available for investigation.

DISCUSSION

In haemagglutination inhibition experiments M and N glycoproteins were more inhibitory than the corresponding glycopeptides. In the RIA for M antigen the steeper inhibition slope obtained for glycoprotein than for glycopeptide suggests that rabbit anti-M has a higher affinity for the glycoprotein (Berson & Yalow, 1968). Affinity is affected by valency of binding (Hornick & Karush, 1972) and the observed difference may be due to multivalent binding to aggregated glycoprotein (Kirschbaum & Springer, 1975). Dahr *et al.*, (1977) and Wasniowska *et al.* (1977) have reported that M and N specificities are linked to amino acid polymorphisms at the first and fifth positions from the

N terminus and Dahr & Uhlenbruck (1978) have assigned the MN antigenic determinants to the N terminal fragment of the molecule. There may, therefore, be only one M or N antigenic determinant per molecule precluding multivalent binding to the monomolecular glycopeptide.

The development of a satisfactory radioimmunoassay for the N antigen was hindered by the lack of potent anti-N antibody. Shallower inhibition slopes were obtained in assays for N than for M glycoprotein suggesting that anti-N has a lower affinity for N glycoprotein than has anti-M for M glycoprotein. Inhibition in the N assay obtained by M glycoprotein suggests lack of specificity, but such inhibition may be due to weak N or 'N' activity which is detectable in MM red cells and which may be located in the N-terminal structure of the Ss glycoprotein (Dahr & Uhlenbruck, 1978) which is also present in butanol extracts of stroma (Anstee & Tanner, 1975).

In the RIA for the T antigen, T glycopeptides were about four-fold more inhibitory than T glycoproteins and, in contrast to the M assay, the inhibition slopes for the glycopeptides and glycoproteins were all very similar. The determinant of the T antigen, Galβ(1–3)GalNac, is present within the oligosaccharide chains of glycophorin but the determinant is normally masked by sialic acid. There are fifteen such side chains per glycophorin molecule (reviewed by Marchesi, Furthmayr & Tomita, 1976) and it may be that desialation of these oligosaccharides permits multivalent binding of rabbit anti-T to glycopeptide molecules.

Results obtained with PNA in the Farr assay for the T antigen differed in several respects from the results obtained with RIA. The sensitivity of the Farr assay for asialofetuin was comparable to the radioassay described by Carter & Sharon (1977) but the Farr assay was over 100-fold less sensitive than the RIA for T antigen. Lack of sensitivity may be due to low affinity although inhibition slopes were steep. Alternatively, there may have been a failure of coprecipitation of the lectin with the human IgG which is added as carrier. There are also important differences in specificity in that rabbit anti-T is much less readily inhibited by monosaccharides and by desialated fetuin than is PNA. Uhlenbruck *et al.* (1969) showed that human anti-T agglutinin is much less readily inhibited by Galβ(1–3)GalNAc than is PNA and suggested that anti-T antibody has a larger binding site than PNA. As the disaccharide is attached directly to the peptide chain of glycophorin an antibody binding site which is larger than the disaccharide must accommodate part of the peptide chain. This implies that anti-T antibody may have a higher specificity for membrane glycoprotein than PNA which recognizes the smaller determinant which is also present on certain serum and secreted glycoproteins (Kornfeld & Kornfeld, 1976). RIA would therefore, appear to be the preferred technique for the detection of soluble T antigen shed from cell surfaces.

The development of sensitive assays of the M, N and T blood group antigens which are borne by the major glycoprotein of red cells and many other tissues will facilitate studies of the release of this glycoprotein from normal and pathological cell membranes. The assay may also facilitate the eventual characterization of the M and N antigenic determinants.

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